

Efficient transcription of the larvicidal *cry4Ba* gene from *Bacillus thuringiensis* in transgenic chloroplasts of the green alga *Chlamydomonas reinhardtii*

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ABSTRACT

Unicellular micro-alga *Chlamydomonas reinhardtii* has been recognized as a promising host for expressing recombinant proteins albeit its limited utility due to low levels of heterologous protein expression. Here, transcription of the 3.4-kb mosquito-larvicidal *cry4Ba* gene from *Bacillus thuringiensis* in transgenic *C. reinhardtii* chloroplasts under control of the promoter and 5'-untranslated region of photosynthetic *psbA* gene was accomplished. Inverted repeats in chloroplast genomes of the host strain with deleted endogenous *psbA* genes were selected as recombination targets. Two transformant lines were obtained by dual-phenotypic screening via exhibition of resistance to spectinomycin and restoration of photosynthetic activity. Stable and site-specific integration of intact *cry4Ba* and *psbA* genes into chloroplast genomes found in both transgenic lines implied homoplasmy of organelle populations. Achievement in co-transcription of *cry4Ba* and *psbA* transgenes revealed by RT-PCR and Northern blot analyses demonstrates the sufficiency of this system's transcription machinery, offering the further innovation for insecticidal protein production.

Keywords: *Chlamydomonas reinhardtii*; Chloroplast Transformation; Inverted Repeats; *Bt-cry4Ba* Transcript; *psbA* Promoter

1. INTRODUCTION

Cry4Ba δ -endotoxin, one of the four major insecticidal proteins produced from *Bacillus thuringiensis* subsp.

israelensis (*Bti*), is specifically toxic against mosquito larvae of *Aedes* and *Anopheles* species that are vectors of widespread deadly contagious diseases, including dengue-viral hemorrhagic fevers, viral encephalitis and malaria (for reviews, see [1,2]). Achievement of over-expression of the recombinant *Bti-cry4Ba* toxin was at first reported in *Escherichia coli* [3]. A further effort was made until that time to manipulate this toxin into the cyanobacterial host, which can exist in an environment more akin to the target larvae [4]. Although the bacterial system holds some potential for producing this insecticidal protein, the expensive cost of industrial-scale production, particularly with regard to bacterial growth media, still hinders wide-scale usage of *Bti* toxins. Protein production in the plant expression system is inherently more cost-effective than that manufactured by bacterial fermentation [5]. However, time-consuming production and pollen-mediated gene flow are still the major drawbacks to this transgenic approach [6].

Single cell micro-alga *Chlamydomonas reinhardtii*, on the other hand, offers a compromise between the bacterial and plant systems in terms of production cost and timescale based on the fact that this microorganism makes use of natural resources, *i.e.*, sunlight and CO₂ for the photosynthetic growth as its generation time is approximately eight hours [7]. Moreover, the time spent from initial transformation to large-scale production is unexpectedly short, approximately six weeks for a flask scale and additional two more months for scaling up to several ten thousand liters [8].

Transgene expression in the chloroplast system thus far is the most attractive technology providing many advantages including the multiple copy number of chloroplast genomes with specific targeted integration *via* homologous recombination. In addition, the problem of gene silencing has not been observed in a transgenic

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chloroplast [9]. Moreover, in the perspective of biosafety, the chloroplast expression system provides an increase in transgene containment by the maternal inheritance as chloroplast genes are only inherited from the maternal parent while paternal chloroplast genes are selectively eliminated in young zygotes [10]. Together with the organism characteristics, these tremendous potentials make the chloroplast of micro-alga *C. reinhardtii* to be a promising bioreactor in terms of economic and commercial needs, although its utility is still limited owing to low levels of heterologous protein expression (for reviews, see [11]).

In order to construct the transformation vector harboring the *cry4Ba* gene to be efficiently expressed in the chloroplast of *C. reinhardtii*, controlling sequences of an algal strong promoter have to be first considered. It is interesting to note that translation of many chloroplast gene transcripts can be stimulated by light as the highest level of light induction has been reported for the photosynthetic *psbA* gene-encoded D1, a core protein of photosystem II [12]. Together with the fact that the combination of promoter/5'-UTR of *psbA* gene plays an important role in defining the levels of accumulation of recombinant proteins, whereas the identity of the 3'-UTR has little or no effect [13]. We therefore selected this powerful light-enhanced promoter in combination with its analogous 5'-UTR sequence to investigate the pliability of *C. reinhardtii* chloroplasts as biological factories capable of producing the prokaryotic *cry4Ba* gene product, even if there is still very limited knowledge on the translation of heterologous transcripts in these algal chloroplasts [11].

2. MATERIALS AND METHODS

2.1. Construction of *C. reinhardtii* Chloroplast Transformation Vectors

A transformation vector containing an antibiotic selection marker (*aadA*, encoding aminoglycoside adenyltransferase which confers resistance to spectinomycin treatment) and a photosynthetic gene (*psbA*) was constructed for screening transformants *via* both spectinomycin resistance and photosynthetic complementation. A right-arm recombination fragment (RF, NCBI_005353, bases 146,986 to 148,500), amplified from *C. reinhardtii* chloroplast genomes by RF-f and RF-r primers (**Table 1**), was introduced into pBluescriptII KS+ (Invitrogen) at *SalI/ApaI* sites, generating pCH1 (**Figure 1**, left panel).

Concurrently, an intermediary plasmid (p4Bds) containing the *cry4Ba* expression cassette was constructed (**Figure 1**, middle panel). A 3.4-kb *EcoRI* (fill-in with T4 DNA polymerase)/*XbaI* fragment of *cry4Ba* from pMU-388 was ligated with a 3.5-kb *NcoI* (fill-in)/*XbaI* fragment of linearized p546 (the chloroplast expression vector, as

Table 1. Primers used in manipulation and verification of the *cry4Ba* transgene in *C. reinhardtii* chloroplasts.

Construction primer ^a	
RF-f	5'-GGCC <u>GT<u>CGAC</u></u> CGCTAGCAATATCTGATGGTAC-3'
RF-r	5'-GGCC <u>GGG<u>CCCC</u></u> GAATCTCAGTTCTAGTGCTAG-3'
LF-f	5'-GCC <u>CCGCGGAC</u> CGTTAGTCGATATTATACAC-3'
<i>rbcL</i> -3'UTR-r	5'- <u>CCCGCGGTGGCGGCCG</u> CTC-3'
Mutagenesis primer ^b	
pUC18ds-f	5'-GAGCTCGT <u>CGA</u> CGTAATCATGG-3'
pUC18ds-r	3'-GGCTCGAGC <u>AGCT</u> GCATTAG-5'
Verification primer	
L1	5'-CTAATGTCAAATCAATCTGTAAATGC-3'
L2	5'-GCTCTAGATTAGTTGTTTGGACTAGAG-3'
R1	5'-TCACTCGTTCATGCAAATTAATTCAATGC-3'
R2	5'-CTTGGTGCTCTTTGCTCAGTTGGAC-3'
4B-946	5'-GAACATCTCGTGTAAGTGCTGC-3'
4B-ORF	5'-ATGAATTCAGGCTATCCGTTAGCG-3'
<i>psbA</i> -f	5'-ATGACAGCAATTTTAGAACG-3'
<i>aadA</i> -f	5'-AAGTCACCATTGTTGTGCACG-3'
<i>aadA</i> -r	5'-GACGGGCTGATACTGGGC-3'

^aRelevant restriction endonuclease sites are underlined and designated above.

^bHighlighted letters in the mutagenic primers indicate the mutated nucleotides with the corresponding original residues shown above, as *EcoRI* site (GAATTC) was changed to *SalI* site (GTCGAC).

was obtained from the Chlamydomonas Resource Center, www.chlamycollection.org) which contains the promoter, 5'-UTR and 3'-UTR of *psbA*. In order to facilitate ligation of the *psbA-cry4Ba* expression cassette into the chloroplast transformation vector, the flanking restriction sites were changed to *SalI* by subcloning into pUC18ds in which the original pUC18 *EcoRI* site was mutagenized to *SalI* (*EcoRI*Δ*SalI*) *via* PCR-based mutagenesis (Stratagene) using the pUC18ds primer set (**Table 1**). In this process, a 4.2-kb *KpnI* (fill-in)/*SacI* fragment of the *psbA-cry4Ba* expression cassette was ligated with the 2.6-kb *SmaI/SacI* fragment of plasmid pUC18ds generating p4Bds. Subsequently, the *cry4Ba* expression cassette from p4Bds was excised and ligated to the *SalI* site of pCH1, yielding pCH2 (**Figure 1**, middle panel).

Finally, a 4.5-kb fragment, composed respectively of a left-arm recombination fragment (LF, NCBI_005353, bases 137,761 to 138,969) and two selectable expression cassettes consisting of the photosynthetic *psbA*-cDNA and the antibiotic-resistant gene, *aadA*, was amplified

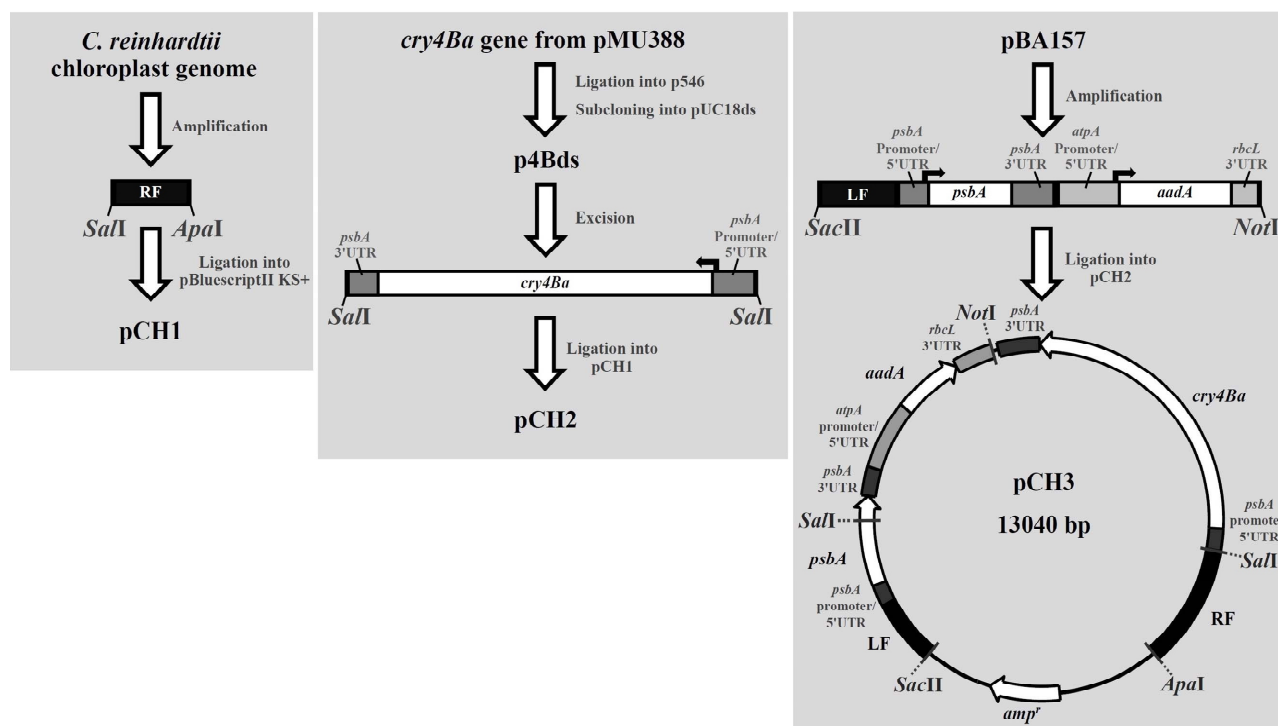


Figure 1. Schematic diagram showing strategy for constructing *C. reinhardtii* chloroplast transformation vectors carrying the *cry4Ba* gene. Subcloning of each fragment into the pBluescriptII KS + backbone; the amplified RF fragment (left panel), the excised *cry4Ba* expression cassette fragment (middle panel) and the combined fragment containing LF and two selectable marker cassettes (right panel) were consecutively ligated into the plasmid backbone generating pCH1, pCH2 and pCH3, respectively. *amp^r* denotes the ampicillin-resistance gene. For clarity, only those restriction sites relevant to the construction are shown.

from pBA157 template [14] by using LF-f and *rbcL*/3'-UTR-r primers (Table 1). The amplified product was subsequently introduced into pCH2 at *NotI*/*SacII* sites downstream of the *cry4Ba* expression cassette, generating the 13-kb chloroplast transformation vector, pCH3 (Figure 1, right panel).

2.2. *C. reinhardtii* Strains and Culture Conditions

C. reinhardtii wild-type (CC-400) and non-photosynthetic *psbA*-deleted (CC-744) strains were obtained from the Chlamydomonas Resource Center. Both strains were grown in TAP (Tris-acetate-phosphate) enriched medium, pH 7.0 [15], in a constant-illumination rotary shaker at 100 rpm, 25°C with light intensity of ~50 $\mu\text{mol photons/m}^2\text{-sec}$. Cells at late-log phase were concentrated by centrifugation until reaching an approximate concentration of 10^7 cells/ml. A 5-ml aliquot of the concentrated cells was spread onto a TAP agar plate containing spectinomycin (final concentration of 100 $\mu\text{g/ml}$) and then allowed to air-dry under sterile conditions.

2.3. Chloroplast Transformation in *C. reinhardtii*

Coating of gold particles (0.1 μm , Bio-Rad) with the chloroplast transformation vector (pCH3) was performed

according to a modified version of a previously described procedure [16]. Chloroplast transformation was achieved *via* particle bombardment using a Helium-driven apparatus (a PDS-1000/He, Bio-Rad), delivered at 900-psi pressure under 25-inches Hg vacuum and 9-cm target distance. After screening via spectinomycin resistance conferred by *aadA* expression cassette [17], *C. reinhardtii* transformant lines were subsequently verified for the recovery of photosynthesis by growing under high-salt (HS) minimal medium [18].

2.4. Genomic DNA Extraction and PCR Identification of Specific Integration

Total genomic DNA was extracted from *C. reinhardtii* cells using the CTAB (cetyltrimethylammonium bromide)-based protocol which was modified from the method of Murray and Thompson [19]. Briefly, after the cell lysate was treated with proteinase K (~0.20 mg/ml, Invitrogen) in Tris-EDTA buffer (pH 8.0) containing 6% SDS at room temperature (25°C) for 15 min, the supernatant was separated by centrifugation and then incubated with DNA extraction buffer (10% CTAB, 0.7 M NaCl) at 65°C for 10 min. The CTAB-extracted DNA was treated with phenol/chloroform/isoamylalcohol (25:24:1) extraction followed with two-time chloroform extraction and

was recovered from aqueous phase by isopropanol precipitation.

PCR reactions (25 μ l) containing total genomic DNA template (50 ng) and a pair of specific primers (5 pmol), *psbA-f/L2*, *aadA-f/aadA-r* and R1/4B-ORF (**Table 1**) in respectively generating products of recombinant 1.0-kb *psbA*, 0.4-kb *aadA* and 3.4-kb *cry4Ba* fragments, were initially performed. Specific localization of the delivered transplastomic genes was further verified by additional reactions employing two pairs of primers (*i.e.* L1/L2 and R1/R2, see **Table 1**) by which the generated PCR products would span left and right recombination targets, respectively. PCR reactions were performed for 30 reaction cycles (98°C, 55°C, 72°C) using Phusion DNA polymerase (Thermo Scientific).

2.5. Semi-Quantitative RT-PCR Analysis

Total RNA was extracted from *C. reinhardtii* cells using the RNeasy plant mini kit (Qiagen) and then subjected to DNase treatment (RQ1 RNase-free DNase, Promega). 250 ng of DNA-free RNA and 10 μ M of *cry4Ba*-specific R1 primer were used in reverse transcription reaction catalyzed by SuperScriptTM II reverse transcriptase (Invitrogen) in the presence of RNase inhibitor (RNase-OUTTM, Invitrogen). 1 μ l of a 1:10 dilution of respective cDNA was used as template for the subsequent PCR reaction using primeSTAR Max DNA polymerase (Takara) with 4B-ORF and R1 primers (see **Table 1**) that bind specifically to start and stop codons of the *cry4Ba* gene, respectively.

2.6. Northern Hybridization Analysis

Large amounts of total RNA from chloroplasts were achieved by means of the ATA (aurintricarboxylic acid) large-scale RNA extraction method [20]. For Northern blotting, probes specific to *cry4Ba* (4B probe) and *psbA* (D1 probe) which are labeled with digoxigenin-11-dUTP (DIG, Roche) were PCR amplified by using primer sets for 4B (4B-946/4B-ORF) and D1 (*psbA-f/psbA-r*) probes, respectively (**Table 1**). After separation on denaturing formaldehyde/agarose gel, RNA was transferred onto a nylon membrane and then hybridized with DIG probe. Target fragments were localized by Anti-DIG (Roche) linked to alkaline phosphatase (AP) and light emission from the enzymatic dephosphorylation of chemiluminescent substrate (CDP-Star, Roche) was detected by X-ray film.

2.7. Protein Extraction and Western Blotting

Total cell and chloroplast protein extracts were prepared as previously described [21]. For Western blot analysis, proteins were fractionated on 10% SDS-PAGE, trans-

ferred to a nitrocellulose membrane and probed with the specific primary monoclonal antibody (2F-1H2 mouse antibody against the Cry4Ba-domain III fragment [22]. ECL (enhanced chemiluminescent) immunoblotting system (Pierce) was used to detect immunoreactive proteins with AP-conjugated goat anti-mouse IgG secondary antibodies.

3. RESULTS AND DISCUSSION

3.1. Establishment of Stable Chloroplast Transformant Lines

To promote *cry4Ba* gene expression in the *C. reinhardtii* chloroplast, the 3.4-kb *cry4Ba* coding sequence was incorporated between the *psbA* promoter/5'-UTR and the 3'-UTR. As with the *Bti-cry4Ba* gene, the *psbA* transgene is also transcriptionally regulated by the identical promoter and terminator sequences (**Figure 1**, right panel). In order to prevent the problem of deletion-looping out recombination [23], *cry4Ba* and *psbA* expression cassettes were assembled in the reverse orientation in the chloroplast transformation vector-pCH3 (**Figure 1**, right panel). It should be noted that both expression cassettes were placed between two integration regions which derived from sequences located within the inverted repeat regions of the *C. reinhardtii* chloroplast genome (**Figure 1**, right panel). Double copies of transgene integration into the cognate sequence in the chloroplast genome were thus expected.

Dissimilar to the gene expression of both *cry4Ba* and *psbA* transgenes, control of expression the bacterial *aadA* gene via the combination of *atpA* promoter/5'-UTR and *rbcL* 3'-UTR was also chosen instead since its analogous expression cassette has been successfully employed for this recombinant protein expression in *C. reinhardtii* chloroplasts [14].

Upon initial screening for the *C. reinhardtii* transgenic lines via resistance to spectinomycin treatment, only a few putative lines were recovered. The low efficiency of this chloroplast transformation may possibly be due to the fact in *C. reinhardtii* that recombination events within the inverted repeats of chloroplast genomes occur preferentially inside the 0.7-kb sequence spanning immediately the 3'-end of *psbA* gene known as hotspots [24]. Given that the mutant host strain used in our work was originally knocked out the whole set of *psbA* gene including the 0.7-kb hotspots, the remaining regions might therefore deteriorate the frequency of recombination events and hence decrease in the number of transgenic lines.

Further phenotypic recombinant screening revealed that two transformant lines, T₁ and T₂, showed the recovery of photosynthetic activity by means of scalability in HS minimal medium. It is noteworthy that the benefits

of additional screening *via* photosynthetic *psbA* gene restoration would be very helpful in ruling out these transformant lines that may have resulted from spontaneous mutation induced by frequent drug screening [25]. It is also interesting to note that both spectinomycin-resistance and restored-photosynthetic phenotypes for these two lines remained stable since both T₁ and T₂ lines have been monthly subcultured for more than a year on fresh TAP agar containing antibiotic spectinomycin. This indicates that the transformation vector was stably integrated into the chloroplast genome given that the co-existing plastid vector would become genetically incompatible and be eventually eliminated [26].

3.2. Specific Integration of Intact Transgenes in Chloroplast Genome and Homoplasmy

Upon initial PCR identification *via* individual transgene-specific primers, single bands of each corresponding gene, 1.0-kb *psbA*, 0.4-kb *aadA* and 3.4-kb *cry4Ba*, were amplified from the total genomic DNA of both lines (T₁ and T₂) as shown in **Figure 2(a)**. To further identify whether the transgenes are specifically integrated into the target inverted repeats of chloroplast genomes, two pairs

of specific PCR primers, L1/L2 and R1/R2 which respectively bind to the *psbA* and *cry4Ba* transgenes, and to their corresponding adjacent endogenous chloroplast genes, were employed (**Figure 2(b)**). As can be seen that a 2.3-kb band was produced from the L1/L2 amplification by which the L1 primer annealed to the endogenous chloroplast *chlN* gene and the L2 primer annealed to the 3'-end of *psbA* transgene. In addition, a 5.1-kb band was amplified from the annealing of R1 and R2 primers to the 5'-end of the *cry4Ba* transgene and the endogenous chloroplast *rrn5* (**Figure 2(c)**). These results indicate that both transgenes were entirely integrated into *C. reinhardtii* chloroplast genomes, further suggesting that the two identical *psbA* promoter sequences used in both *cry4Ba* and *psbA* expression cassettes did not cause a deletion-looping out phenomenon.

As mentioned earlier that the two transgenic lines stably exhibited both spectinomycin-resistance and recovered-photosynthetic phenotypes that are offered respectively by *aadA* and *psbA* transgenes. Therefore, this phenotypic stability along with the gene integrity of the chloroplast transgenes would most likely lead to homoplasmy of organelle populations as incompatible co-existing plastid molecules would be segregated to yield

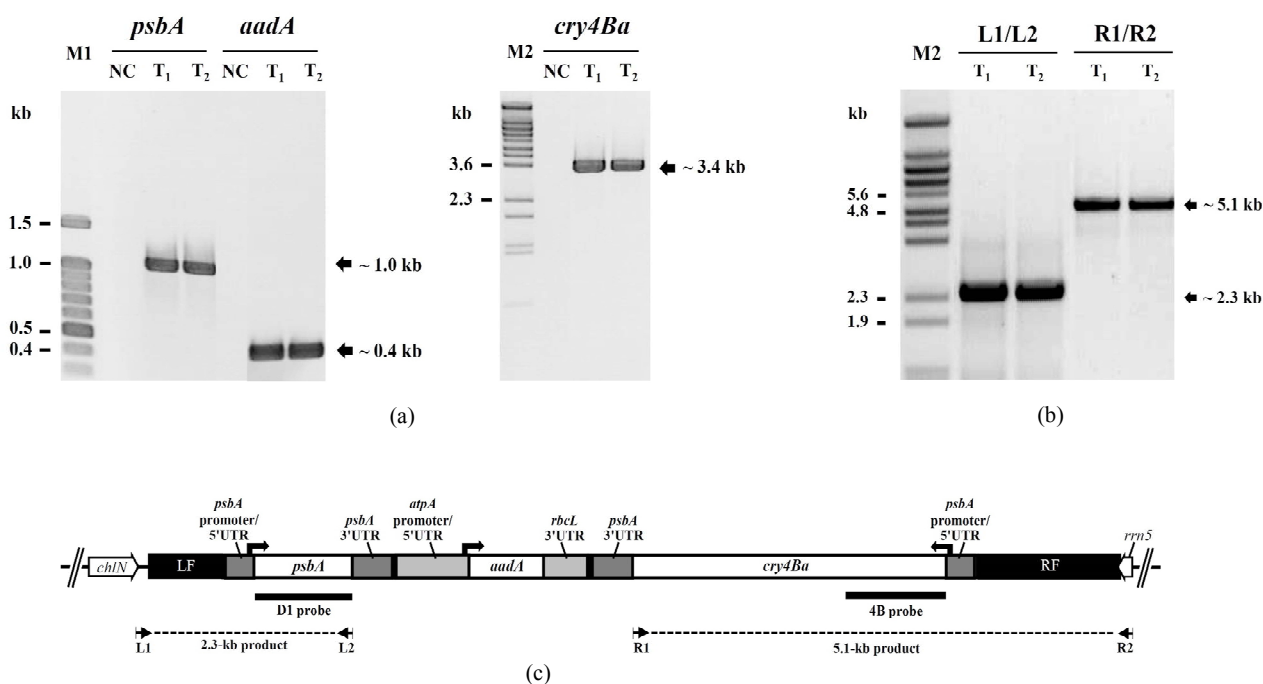


Figure 2. Transgene integration into *C. reinhardtii* chloroplast genome. (a) PCR analysis of all three recombination fragments (1.0-kb *psbA*, 0.4-kb *aadA* and 3.4-kb *cry4Ba*, as indicated above each corresponding figures) amplified from total genomic DNA extracted from both transformant lines, T₁ and T₂. NC denotes a non-transformant line CC-744 used as a negative control. Only those sizes of standard markers, M1 (100-bp DNA ladder) and M2 (*Bst*EII-digested λ DNA) relevant to amplified products are shown; (b) Schematic representation of the expected left inverted repeat (IR_b) region in the transgenic chloroplast genome as a result from specific integration of the pCH3 transformation vector. Binding sites of individual primer pairs, L1/L2 and R1/R2, and their correlated spanning products are shown under the map. Corresponding locations of D1 and 4B probes used in Northern blotting are denoted by black bars; (c) PCR identification of specific integration of both *psbA* and *cry4Ba* transgenes, showing 2.3-kb and 5.1-kb products amplified from both transformant lines, using L1/L2 and R1/R2 primer sets, respectively.

homoplasmic isolates after a long period repeatedly sub-culture under selective conditions [27].

3.3. High Yield and Integrity of *cry4Ba* Transcripts in Transgenic Chloroplasts

To verify that the 3.4-kb *cry4Ba* transgene which was wholly integrated in the chloroplast genome and placed under the photosynthetic *psbA* promoter was ably transcribed, RT-PCR and Northern blotting were performed. For RT-PCR analysis, the results revealed that the *cry4Ba* gene in both T₁ and T₂ lines is efficiently and completely transcribed as indicated by the presence of a 3.4-kb high-yield product comparable to that directly amplified from the control vector template (Figure 3). It is noteworthy that this 3.4-kb corresponding band was absent in the RT-negative control, confirming that the RT-PCR product was derived from the DNA-free *cry4Ba*-mRNA template. In addition, this single full-length RT-PCR product of 3.4 kb could be an indicative of the transcript integrity.

Consistent with the RT-PCR results, Northern blotting (NB) also revealed a major ECL-positive band of ~3.4 kb in the total RNA extracts of both T₁ and T₂ lines when hybridized with the specific *cry4Ba* probe (Figure 4, right panel), further strengthening the efficient transcription of the recombinant *cry4Ba* gene in transgenic chloroplasts. It is worth mentioning that an intense NB band of ~1.0-kb was specifically revealed in both transgenic lines when probed with the *psbA*-D1 fragment (Figure 4, left panel). The data thus established that algal chloroplasts have adequacy of basal transcription factors utilized to efficiently co-transcribe the *cry4Ba* and *psbA* transgenes driven by each individual *psbA*-controlling cassette.

3.4. Deficient Production of the Recombinant *cry4Ba* Protein in Transgenic Chloroplasts

Attempts were also made to examine the expression of

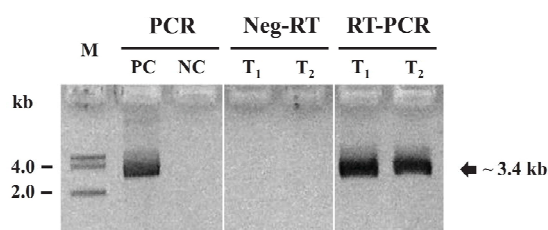


Figure 3. RT-PCR analysis of *cry4Ba* transgenic transcripts in both *C. reinhardtii* transgenic lines, T₁ and T₂. PC and NC denote positive and negative PCR control reactions, respectively, as PC contains the pCH3 template while NC omits a corresponding template. Neg-RT represents a control RT-PCR reaction with reverse transcriptase enzyme omitted. Relevant sizes of DNA marker (M, Gene ladder fast 2) are shown.

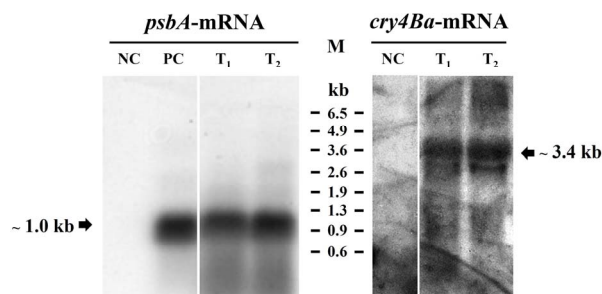


Figure 4. ECL-autoradiograms of Northern blots containing total RNA extracted from *C. reinhardtii* transgenic lines, T₁ and T₂, showing the accumulation of the two transgenic transcripts, *i.e.* ~3.4-kb *cry4Ba* (right panel, as detected by 4B probe) and ~1.0-kb *psbA* (left panel, as detected by D1 probe). NC denotes a negative control as RNA was extracted from strain CC-744. Total RNA from the CC-400 wild-type strain was used as a positive control for the synthesis of an endogenous *psbA* transcript.

the 130-kDa Cry4Ba recombinant protein in transgenic chloroplasts of T₁ and T₂ lines which show efficient transcription of this intact transcript as demonstrated earlier (see Figure 3). As assessed by ECL immunoblotting probed with the specific Cry4Ba-domain III monoclonal antibody, the results revealed no demonstrable accumulation of this recombinant protein under the conditions used (data not shown). These results therefore make a note of a poor correlation between protein accumulation and high-yield transcript production in the *C. reinhardtii* chloroplast as also noticed previously [28,29]. However, the reasons why this particular heterologous transcript is defectively translated in the transgenic chloroplast are still unclear at present.

A number of studies have revealed that translational efficiency is a rate-limiting step for transgene expression in *C. reinhardtii* chloroplasts, and some have demonstrated that the bias seen in codon usage has a profound effect on recombinant protein accumulation in the algal chloroplasts (for reviews, see [11]). Here, we thus evaluated the codon usage of the *Bti*-Cry4Ba protein-coding gene to check its preference for the utilization of synonymous persistent codons in comparison with that of the *C. reinhardtii* chloroplast genome (www.kazusa.org.jp/codon). Although the patterns of synonymous codon usage in both the bacterial *cry4Ba* transgene and the *C. reinhardtii* chloroplast genome are quite similar as almost all codons ending in A or U are preferred, there is a strong bias in the Cry4Ba-coding gene towards the use of glycine GGG and histidine CAC codons (see Table 2). It is therefore possible that the deficient translation of the high-yield *cry4Ba* transcript in transgenic chloroplasts could perhaps be due to biases seen in glycine and histidine codons used in this recombinant protein-coding gene. Hence, further studies *via* codon optimization of

Table 2. Comparison of the bias usage between the *Bti-cry4Ba* transgene and the *C. reinhardtii* chloroplast genome (Kazusa database, www.kazusa.org.jp/codon) noticed in glycine and histidine codons.

Amino acid/Codon usage	Usage percentage ^a		
	<i>cry4Ba</i> gene	Kazusa database	
Glycine	GGU	27	<u>70</u>
	GGC	10	10
	GGA	29	14
	GGG	<u>34</u>	6
Histidine	CAU	7	<u>53</u>
	CAC	<u>93</u>	47

^aThe most usage frequency of individual codons in each coding sequence is underlined.

this non-native gene are of great interest since a codon-optimized *cry4Ba* gene might be indeed a requirement for improving the heterologous production of the *Cry4Ba* insecticidal protein in *C. reinhardtii* chloroplasts, although some other unmodified *Bt-cry* genes, *i.e.* *cry1Ab*, *cry1Ac* and *cry2Aa2*, have been effectively expressed with high levels of insecticidal protein accumulation in plant chloroplasts (for reviews, see [22]). Nevertheless, another possibility that the *Cry4Ba*-coding sequence *per se*, besides codon bias, might be crucial for translation efficiency in transgenic chloroplasts remains to be investigated.

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